(12) UK Patent Application (19) GB (11) 2 327 224 (13) A

(43) Date of A Publication 20.01.1999

(21) Application No 9814753.1

(22) Date of Filing 07.07.1998

(30) Priority Data
(31) 08890467 (32) 09.07.1997 (33) US

(71) Applicant(s)
Mayo Foundation For Medical Education and Research
(Incorporated in USA - Minnesota)
200 First Street SW, Rochester, Minnesota 55905, United States of America

(72) Inventor(s)
James A Levine
Norman L Eberhardt
Michael D Jensen
Timothy O'Brien

(74) Agent and/or Address for Service
Stevens, Hewlett & Perkins
1 Serjeant's Inn, Fleet Street, LONDON, EC4Y 1LL, United Kingdom

(51) INT CL[6]
A01K 67/027 , C12N 5/06 // C12N 15/86 15/88

(52) UK CL (Edition Q)
C3H HB7T
C6Y Y409 Y501 Y503
U1S S1068 S1303 S1317 S1334

(56) Documents Cited
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Proc.Natl.Acad.Sci.USA 1997,94,6386-6390 Biochimica et Biophysica Acta 1997,1330,8-16

(58) Field of Search
UK CL (Edition P) C3H HB7T
INT CL[6] A01K 67/027 , C12N 5/06 5/10 15/86 15/88
ONLINE: WPI,CLAIMS,DIALOG/BIOTECH

(54) Abstract Title
Gene transfer to adipocytes

(57) An in vivo method of transferring a nucleic acid sequence into an adipocyte is described. The method involves contacting mammalian adipose tissue containing an adipocyte with a transfer vehicle containing the nucleic acid sequence, where the transfer vehicle is capable of entering the adipocyte, and where an expression product is produced from the nucleic acid sequence. Adipocytes containing such transfer vehicles, and adipose tissue containing such adipocytes, as well as methods of altering adipocyte growth and metabolism in mammals by contacting adipose tissue of mammals with such transfer vehicles and methods of identifying nucleic acid sequences involved in adipocyte growth and metabolism, are also described.

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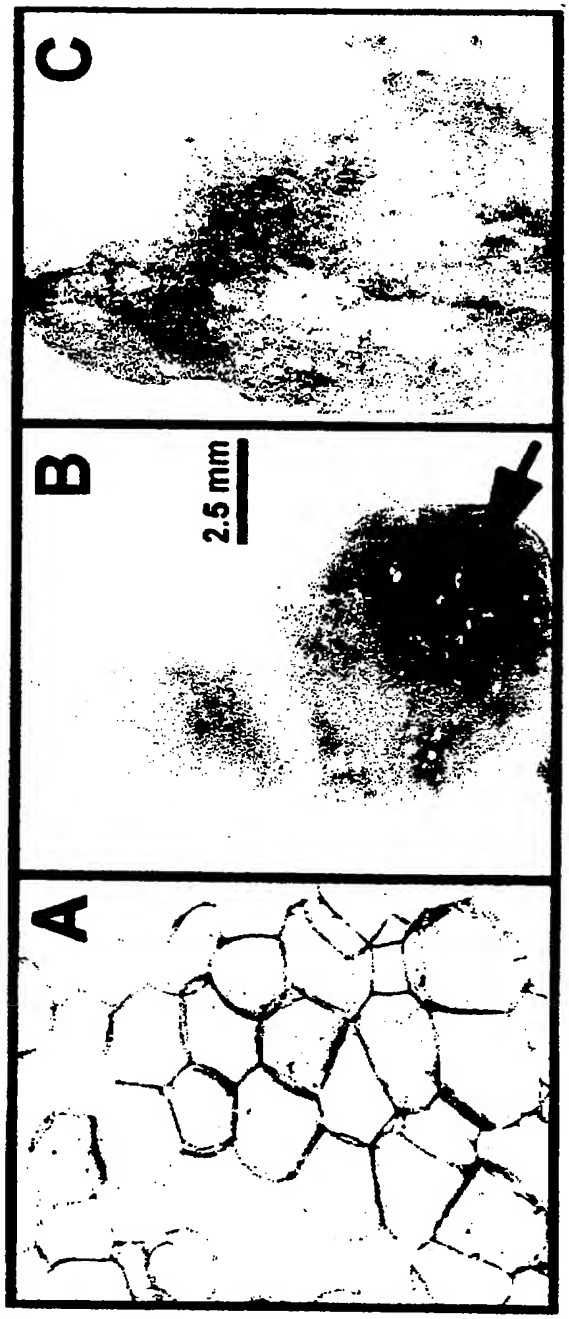


Fig. 1

Fig.2A

Fig.2B

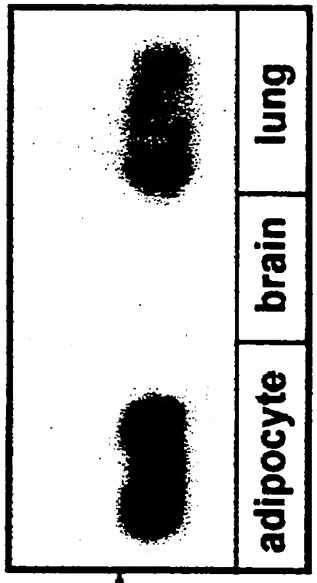
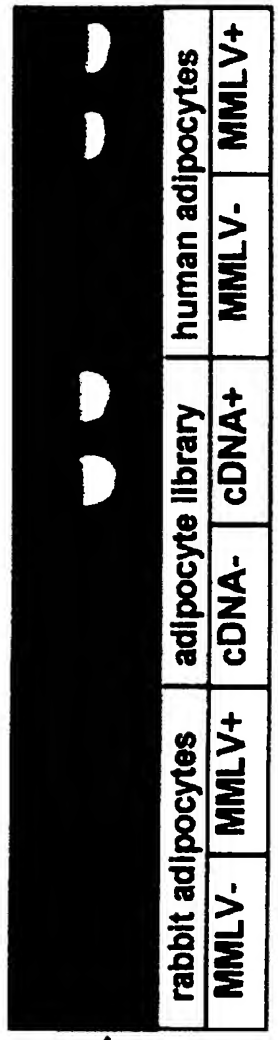


Fig.2C



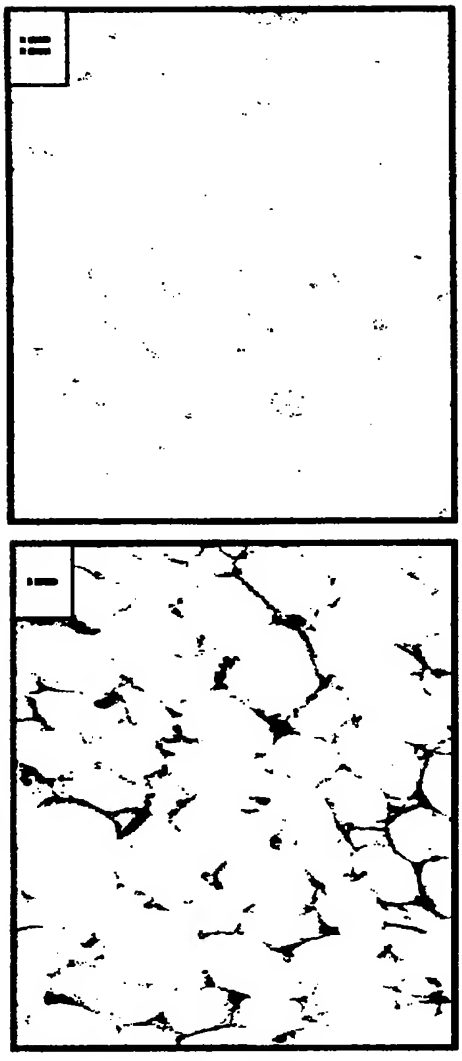


Fig. 2D

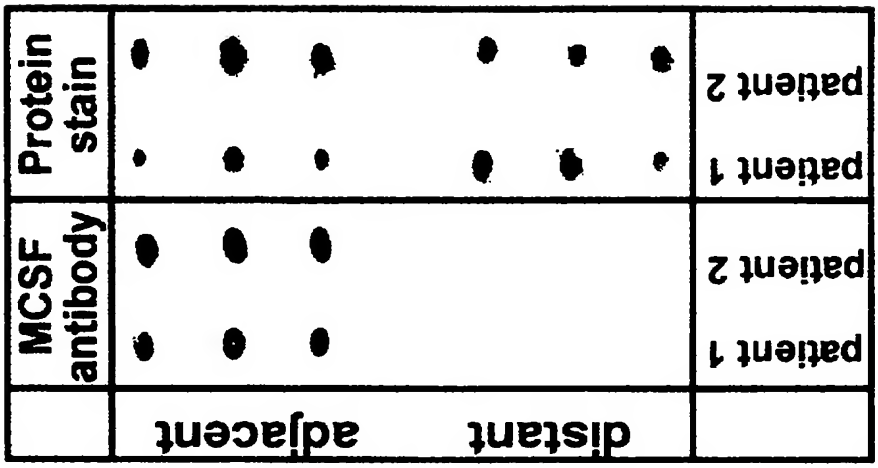

distant
adjacent
MCSF antibody
patient 1
patient 2
Protein stain
patient 1
patient 2

Fig. 2F

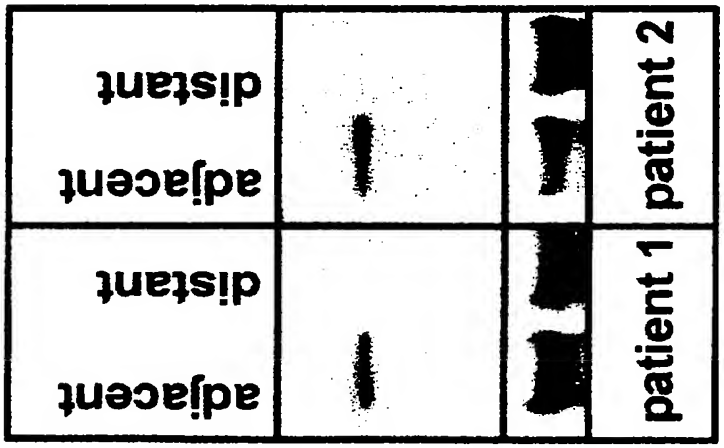

adjacent
distant
adjacent
distant
patient 1
patient 2
MCSF
28S

Fig. 2E

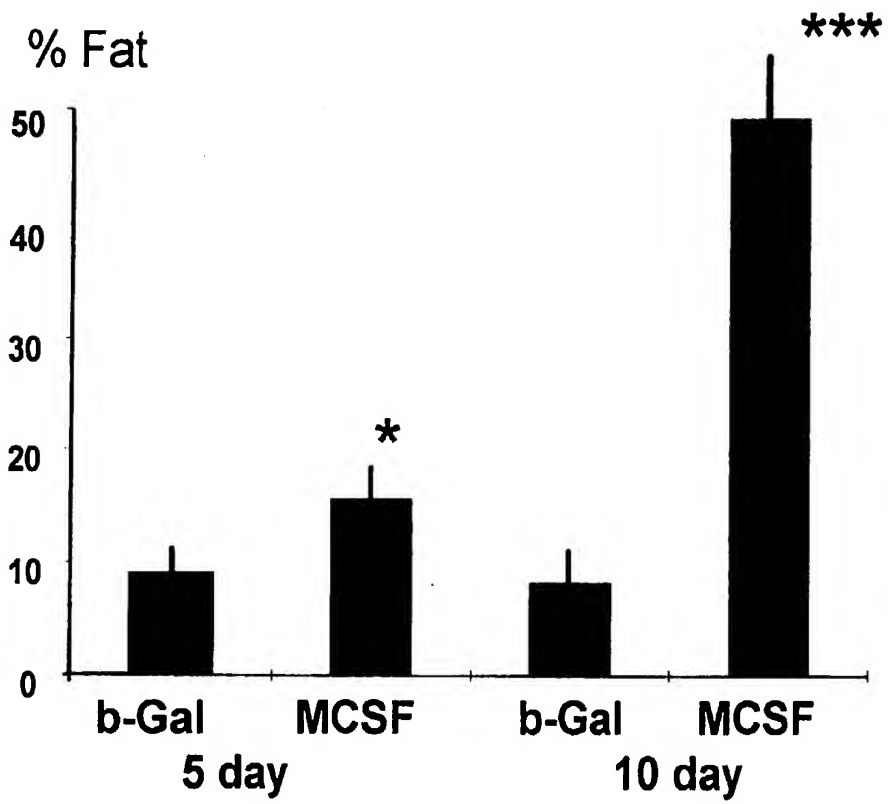

Percent fat in 2 X 2 cm squares
% Fat
50
40
30
20
10
0
*
***
b-Gal
MCSF
b-Gal
MCSF
5 day
10 day

Fig. 3

Xgal staining of b-Gal innoculated subcutaneous fat, Rabbit 2

Fig.4

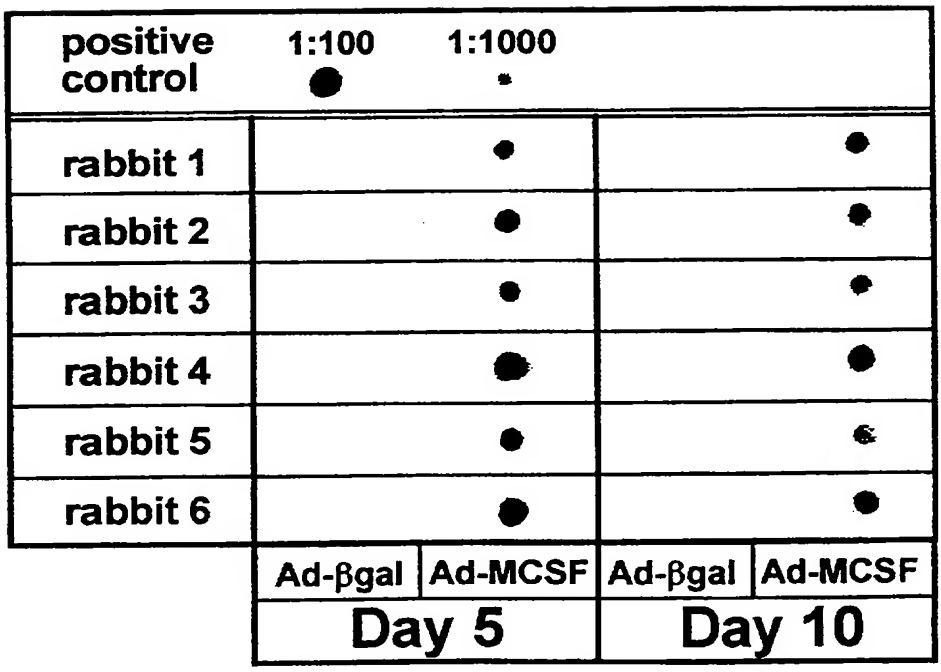

positive control
1:100
1:1000
rabbit 1
rabbit 2
rabbit 3
rabbit 4
rabbit 5
rabbit 6
Ad-βgal
Ad-MCSF
Ad-βgal
Ad-MCSF
Day 5
Day 10

Fig. 5

Immunostaining of Day 5, Rabbit 2, MCSF innoculated subcutaneous fat

Fig. 6A

Immunostaining of Day 5, Rabbit 2,
b-Gal innoculated subcutaneous fat

Fig. 6B

Fig. 7a

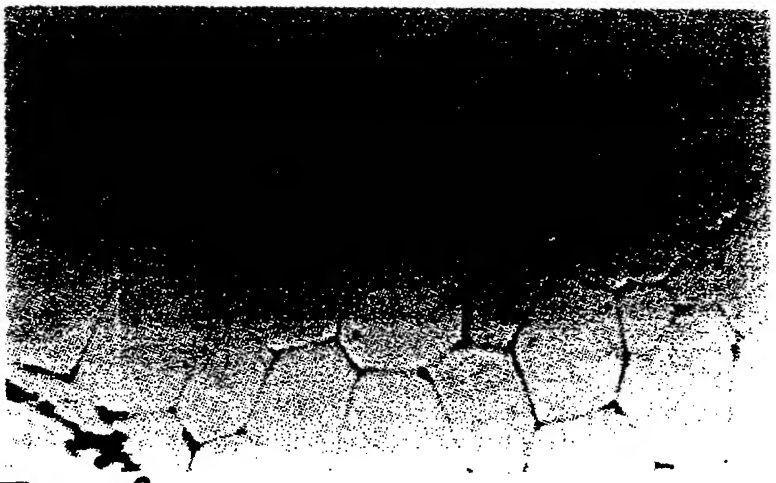
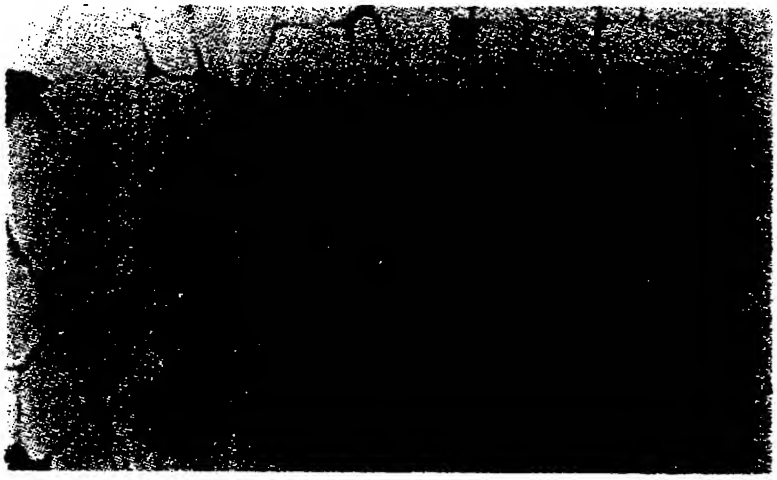
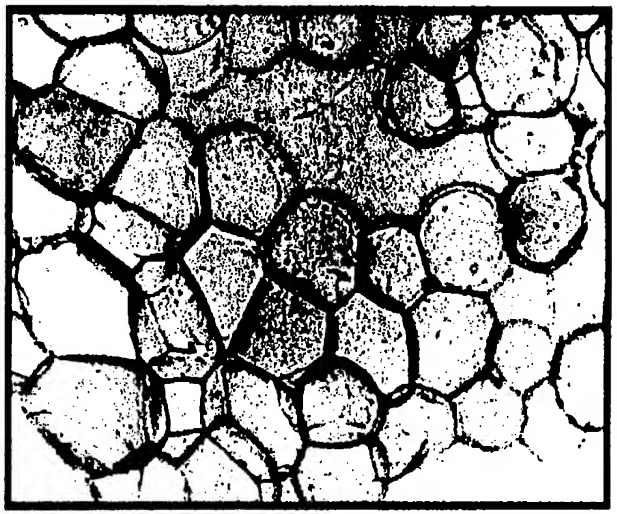


Fig. 7b

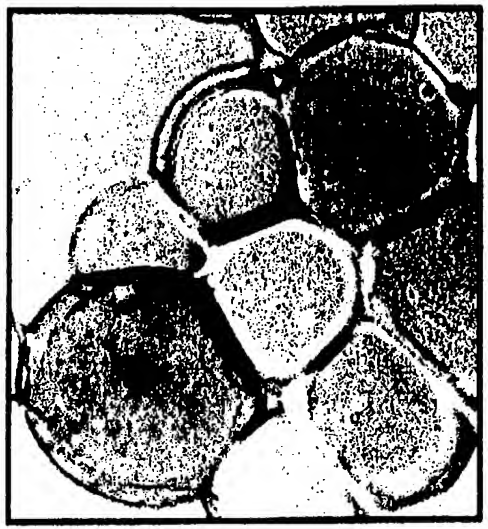


Proliferative marker (MIB-1) in (a) b-Gal transduced and (b) MCSF transduced subcutaneous fat at 10 Days, Rabbit 4

100 X



200 X



human adipocytes exposed to Ad-βgal *in vitro*

Fig. 8

Xgal stained adipocyte from b-gal-adenovirus innoculated human adipose tissue ex vivo

Fig. 9

GENE TRANSFER TO ADIPOCYTES

Background of the Invention

This invention relates to methods of transferring genetic material into adipocytes and to the screening of genes, and the expression products of such genes, potentially involved in the regulation of adipocyte growth and metabolism.

Obesity may be defined as the presence of excess adipose tissue. Obesity affects between 30-50% of the North American population and a substantial percentage of the population worldwide. The effects of obesity, e.g. non-insulin dependent diabetes, coronary artery disease, and hypertension, are estimated to have resulted in $45.8 billion in direct costs and an additional $23 billion in indirect costs from missed work in 1990. Gibbs, W.W., Aug. 1996, Scientific American, p. 88-94. In addition, an estimated $30 billion is spent on weight loss products each year. Gura, T., 1997, Science, 275: 751-753.

Changes in the occurrence of obesity over time, within populations and individuals, suggests that environmental factors play a role in the development of obesity. Genetic factors are also known to exert a strong influence in obesity. Body mass indexes, one measure of obesity, are usually similar among family members. The body mass index of adopted children, for example, resembles their biological parents, not the adopted parents. Detailed studies of monozygotic twins show that other measures of obesity, such as subcutaneous skinfold thickness, fat mass, and fat-free mass, are also similar. Sørensen, T.I.A. 1995, Metabolism, 44: 4-6, Suppl. 3.

Excess adipose tissue is accumulated through an imbalance between energy intake and energy expenditure. Obesity therapies traditionally have manipulated dietary

intake to correct this imbalance. Various obesity therapy programs exist that reduce caloric intake through patient education, but have a failure rate approximating 90% after five or ten years follow up. Other programs include formulas for nutritional sustenance to either supplement or replace normal food. Typically, weight is regained once the patient stops taking the supplement. Pi-Sunyer, F.X., 1996, Clinical Therapeutics, 18(6):1006-1035.

Most current pharmacological therapies for obesity target the hypothalamic region of the brain, resulting in significant weight loss. Once therapy is discontinued, however, patients frequently regain the lost weight. Davis, R., 1996, Drugs, 52(5):696-724; Ryan, D.H., 1996, Endocrinology & Metabolism Clinics of North America, 25(4):989-1004. The cost of such therapies is high, with physician supervision and life-long prescriptions required. In addition, these therapies can produce serious untoward side effects. The use of appetite suppressant drugs, especially derivatives of fenfluramine, is associated with an increased risk of primary pulmonary hypertension in patients taking the drugs for more than three months. Abenhaim, L. et al. 1996, N. Engl. J. Med., 335: 609-616. Other side effects, including cardiac rhythm abnormalities and altered sensorium, have been described with such therapies. Davis, R., 1996, Drugs, 52(5):696-724. B3 adrenoreceptor agonists have also been evaluated as potential therapeutics for obesity, but have been disappointing in human studies due to poor side effect profile and unproven long-term efficacy.

Surgery is another possible treatment for obesity. Gastric bypass surgery attempts to limit a patient's food intake by reducing stomach size, while maintaining normal digestion and absorption. A high surgical mortality rate

(5%) and post-operative complications limit this treatment to morbidly obese patients.

Little attention has been given to elucidating genetic regulation in adipocytes or adipose tissue, the end organ of obesity, and the role such regulation might play in the onset or prevention of obesity. Because therapeutic intervention in obesity has focused on the central nervous system, there has been little development of therapies targeted to adipocytes or adipose tissue. Because adipocytes and adipose tissue are the sites of lipid storage and complex lipid metabolism, it would be useful to identify genes that are implicated in the regulation of adipocyte growth and metabolism and in obesity, and to develop a model system useful in elucidating the roles of such genes in adipocyte regulation. Such a model system may provide the basis for therapies directed to adipose tissue in the treatment or prevention of obesity.

Summary of the Invention

The present invention provides a useful model system for the study of genetic regulation of adipocyte growth and metabolism, and for the study of the biological effects of protein expression products of genes or nucleic acid sequences of interest. As disclosed herein, the present invention provides a method of transferring a nucleic acid sequence into an adipocyte such that an expression product of the nucleic acid sequence is produced in the adipocyte. The expression product may then be correlated with a physiologic effect, such as an increase or decrease in fat storage. The present invention also provides methods of altering the growth and/or metabolism of adipocytes in mammals, methods that may be utilized therapeutically to treat obesity or to promote fat storage, depending on the needs of a particular patient.

In one aspect, the invention features an in vivo method of transferring a nucleic acid sequence into an adipocyte. The method involves contacting adipose tissue of a mammal with a transfer vehicle containing the nucleic acid sequence. The transfer vehicle, e.g. an adenovirus or liposome, is capable of entering the adipocyte and contains a nucleic acid sequence. An expression product is produced from the nucleic acid sequence when transferred into the adipocyte. The method may further include detecting the presence of the expression product and correlating the presence of the expression product with a physiologic effect in the adipocyte. One example of a nucleic acid sequence shown herein to be transferred in vivo into adipocytes and to produce a physiologic effect, is a nucleic acid sequence encoding macrophage colony stimulating factor (MCSF).

The nucleic acid sequence that is transferred into adipocytes in accordance with the invention may be a sequence known to be differentially regulated in adipocytes, including a sequence known to be differentially regulated in white versus brown adipocytes. Uncoupling protein I (UCPI) or a portion thereof is one example of a nucleic acid sequence known to be differentially regulated in white versus brown adipocytes.

In another aspect, the invention features adipocytes containing a transfer vehicle with a nucleic acid sequence that is differentially regulated in adipocytes, and further features adipose tissue isolated from a mammal, wherein such adipose tissue contains at least one adipocyte harboring a transfer vehicle containing a nucleic acid sequence that is differentially regulated in adipocytes.

In still another aspect, the invention features a method of altering adipocyte growth and metabolic

regulation in a mammal. This method includes the step of contacting adipose tissue of the mammal with an effective amount of a transfer vehicle containing a nucleic acid sequence, wherein the transfer vehicle is capable of entering an adipocyte of the mammal. Production of an expression product from the nucleic acid sequence in the adipocyte alters the growth and metabolic regulation of the adipocyte. Suitable nucleic acid sequences include genes or portions thereof that are differentially regulated in white versus brown adipocytes. An illustrative example of such a nucleic acid sequence is UCPI or a portion thereof. This method provides the basis for therapuetic intervention to treat obesity or promote adipose tissue growth or proliferation in a patient.

In another aspect, the invention relates to a method for identifying nucleic acid expression products that alter growth and metabolic regulation in an adipocyte. A candidate nucleic acid sequence is transferred into mammalian adipose tissue. A product is expressed from the nucleic acid sequence and changes in adipocyte growth and metabolic regulation are monitored. Examining a change in adipose tissue weight is one preferred method by which adipocyte growth and metabolic regulation may be monitored.

As described herein, the present invention has several advantages. The model system provided by the present invention allows nucleic acid sequences that may be involved in adipocyte metabolic and growth regulation to be screened <u>in vivo</u> in the adipocyte and adipose tissue, the end-organ of obesity. <u>In vivo</u> screening permits the evaluation of candidate nucleic acid sequences in a physiological setting where the effects of expression products are not isolated from other physiologic and biochemical processes in the mammal, as

they would be in an in vitro culture system. Identification of nucleic acid sequences involved in adipocyte growth and metabolic regulation provides new targets for therapy, either for obesity or for patients suffering from a deficit of adipose tissue or stored lipid.

Brief Description of the Drawing

Figure 1 is a β-galactosidase transfected rabbit fat pad stained with X-gal.

Figure 2A is a Northern blot of human adipocyte total RNA probed with an antisense RNA to MCSF.

Figure 2B is a Western blot of human adipocyte protein.

Figure 2C depicts the amplification of MCSF from rabbit and human adipocytes and a human adipocyte cDNA library.

Figure 2D depicts immunostaining of human MCSF in adipose tissue in the presence (i) and absence (ii) of human MCSF monoclonal antibody.

Figure 2E is a Northern blot of total RNA, extracted from mesenteric adipocytes distant from and adjacent to bowel inflammation, probed with an antisense RNA to MCSF.

Figure 2F is an immunoblot of mesenteric adipocytes distant from and adjacent to bowel inflammation stained with Ponceau S and exposed to MCSF antibody.

Figure 3 is a graph depicting the percent fat in 2x2 cm tissue squares excised from β-galactosidase and MCSF transfected rabbit adipose tissue.

Figure 4 depicts X-gal staining of adipocytes from β-galactosidase transfected rabbit adipose tissue.

Figure 5 is a dot blot of β-galactosidase and MCSF transfected rabbit tissue stained with human MCSF antibody.

Figures 6A and 6B depict the immunostaining of MCSF and β-galactosidase-transfected rabbit adipose tissue.

Figure 7 depicts the immunostaining of β-galactosidase and MCSF transfected rabbit adipose tissue with proliferative marker MIB-1.

Figure 8 depicts X-gal staining of human adipocytes transfected with β-galactosidase *in vitro*.

Figure 9 depicts X-gal staining of human adipose tissue transfected with β-galactosidase *ex vivo*.

Description of the Preferred Embodiments

The invention relates to a method for examining biological effects of nucleic acid expression products in adipocytes and adipose tissue in vivo. In this method, genetic material is transferred to adipocytes in mammalian adipose tissue by contact with a transfer vehicle containing genetic material. After entering the adipocyte, a product is expressed from the genetic material.

Adipocytes, the cells that store lipid, are a potential target for obesity therapy that has not yet been exploited. Because obesity is defined as the presence of excess adipose tissue, this tissue represents the end-organ of the disease. Adipocytes account for over 90% of adipose tissue. Stromal tissue, the other main component, includes fibrous bands that organize adipocytes into localized regions, blood vessels, nerves, and lymphatics. Genetic factors have been implicated in the susceptibility for obesity, suggesting that biological events in the adipocyte such as gene up-regulation or overexpression, mediate or at least reflect the susceptibility to storage of excess calories as fat in adipocytes.

Regional differences in adipose tissue deposition occur in all mammals. In humans, regional adipose tissue

deposition predicts the metabolic morbidity associated with obesity. Consistent with these observations, studies have described marked variability in metabolic activity and gene expression between different fat storage sites, or "depots". There are several lines of evidence suggesting adipose tissue can accumulate in a local fashion.

First, as humans gain weight, pre-existing depots of adipose tissue increase in size. Second, if adipose tissue is surgically removed from a local site, local reaccumulation of adipose tissue occurs at that site. Third, inflammation of the bowel and skin are associated with localized adipose tissue growth around regions of inflammation. Factors responsible for promoting local adipose tissue deposition have not yet been identified.

Adipocytes are found subcutaneously and also in bone marrow. Bone-marrow adipocytes are known to synthesize a number of cytokines, including tumor necrosis factor alpha (TNFα), granulocyte colony stimulating factor (GCSF), macrophage colony stimulating factor (MCSF), and various interleukins. The cytokines are essential for hematopoiesis and terminal differentiation of pluripotent stem cells. Yoshikubo, T. 1994, Blood, 84: 415-420. Histologically, bone marrow and subcutaneous adipose tissue adipocytes are similar, with lipids comprising 95% of the cellular volume.

Subcutaneous adipose tissue was traditionally considered a storage tissue, but is now known to secrete hormones and cytokines as well as to respond to such signals. Weight gain and increased adipocyte mass is accompanied by increased TNF-α expression in subcutaneous adipocytes, whereas weight loss is accompanied by decreased TNF-α expression. Hotamisligil, G.S. et al. 1993, Science, 259: 87-91. Subcutaneous adipocytes also secrete leptin, a hormone involved in a weight control

pathway in rodents. Saladin, R. et al., 1996, Hormone & Metabolic Research, 28(12):638-641.

Two different types of adipocytes, white and brown, constitute white and brown adipose tissue respectively, and are present in mammals. White adipocytes store fat as a single large coalescent lipid droplet and release it later according to nutritional needs. Brown adipocytes store lipid in microdroplets and are a source of heat generation in certain mammals and in human neonates. Uncoupling protein I (UCPI), a 33 kDa protein unique to the mitochondrial membrane of brown adipocytes, uncouples respiration from oxidative phosphorylation to generate heat. Expression of UCPI is regulated by the sympathetic nervous system in response to cold and excess caloric intake. Sears, I.B., 1996, Mol. Cell. Biol., 16(3): 3410-3419.

In one embodiment of this invention, an in vivo method of transferring nucleic acid sequences into adipocytes located within subcutaneous mammalian adipose tissue is provided. The method comprises contacting mammalian adipose tissue with a transfer vehicle that is capable of entering into adipocytes. The transfer vehicle contains a nucleic acid sequence, wherein an expression product is produced from the nucleic acid sequence after transfer into an adipocyte. This method provides a powerful model system for the identification and evaluation of nucleic acid sequences whose expression products alter adipocyte growth and metabolic regulation.

A "transfer vehicle" as used herein refers to any agent that is capable of entering an adipocyte upon contact and depositing nucleic acids within the cell. Nonlimiting examples of such agents include recombinant adenoviruses and liposomes. Recombinant adenoviruses are easily manipulated in the laboratory, do not require proliferation for gene transfer, and rarely integrate

into the host genome. Smith, T.A. et al., 1993, Nat. Genet., 5: 397-402, Spector, D.J., and Samaniego, L.A., 1995, Meth. Mol. Genet., 7: 31-44. Adenoviruses have been used to transduce a variety of cell types, including keratinocytes, hepatocytes, epithelial cells, and, most recently, terminally differentiated adipocytes in vitro. Gnudi, L., 1997, Mol. Endo., 11(1): 67-76, Frevert, E.U. and Kahn, B.B., 1997, Mol. Cell. Biol., 17(1): 190-198.

Liposomes are artificial membrane vesicles that are useful as delivery vehicles in vitro and in vivo. In addition to mammalian cells, liposomes have been used for delivery of nucleic acid sequences in plant, yeast and bacterial cells. The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

A "nucleic acid sequence" as used herein refers to a nucleotide sequence that encodes an entire protein or portions thereof. A nucleic acid sequence of the invention may be in the form of RNA or in the form of DNA, including cDNA, synthetic DNA or genomic DNA. The DNA may be double-stranded or single-stranded. Nucleic acid sequences suitable for this method may include any sequence of interest, and may include sequences from genes known to be differentially regulated in adipocytes of an individual, including from different adipocyte types, such as brown adipocytes found in brown fat and white adipocytes found in white fat. "Differentially regulated" as used herein refers to nucleic acid sequences that are subjected to transcriptional, processing, transport, mRNA stability or translational controls within a cell, leading to different profiles of

expression products in different cells. Nucleic acid sequences known to be differentially regulated in adipocytes include, for example, those encoding lipoprotein lipase, leptin and UCP1.

Examples of methods of contacting mammalian adipose tissue with such a transfer vehicle, in accordance with the invention, are set forth in the Examples below. In general, adipose tissue of the mammal is locally contacted with the transfer vehicle through an injection. Alternative methods of contact, such as dermal contact through a patch or a lotion, could also be utilized. Following contact of the adipose tissue with the transfer vehicle, an expression product is produced from the nucleic acid sequence. Northern blots, for example, may be used to detect the messenger RNA encoding the expression product. In addition, the presence of an expression product may be detected, for example, by Western blotting and immunoblotting. Local contact of the transfer vehicle and the subsequent expression of the nucleic acid expression product allows highly specific biological responses to be mimicked. In addition, local administration may minimize untoward side-effects. After confirmation of expression, the presence of the expression product may be correlated with a biological or physiologic effect such as increased proliferation, growth, or lipid storage.

Adipose tissue and adipocytes comprising a transfer vehicle containing a nucleic acid sequence that is differentially regulated in adipocytes may be obtained from biopsies of transfected tissue. Adipocytes may be maintained in primary culture using standard methods. These adipocytes are useful for analyzing the physiological effects of the expressed product and for screening compounds that may alter the physiological effect.

In another embodiment, a method for altering adipocyte growth and metabolic regulation in a mammal is provided. Adipocyte growth and metabolic regulation may be altered, for example, in an obese subject who needs to decrease adipose mass, or in a subject with diabetes, cancer or AIDS, who needs to increase adipose mass. The method includes administering an effective amount of a transfer vehicle that is capable of entering an adipocyte of a mammal. Preferably, an effective amount of a transfer vehicle transfects a maximum number of targeted cells. Using the methods exemplified below, for example, a transfer vehicle may transfect from at least about 9% to about 30% or more of targeted cells. The transfer vehicle contains a nucleic acid sequence, wherein an expression product is produced from the nucleic acid sequence in the adipocyte and alters the regulation of growth and metabolism in the adipocyte. The nucleic acid sequence may be from a gene known to be differentially regulated in adipocytes. "Alteration of growth and metabolism" refers to increasing or decreasing adipocyte growth, storage, proliferation, and metabolism.

In another aspect, the invention relates to a method for identifying nucleic acid expression products that alter metabolic and growth regulation in an adipocyte. The method includes identifying candidate nucleic acid sequences and transferring the nucleic acid sequences into adipose tissue of a mammal, in accordance with the methods of transfer described above. A candidate nucleic acid sequence may be identified and selected from any source, and a transfer vehicle containing such a candidate nucleic acid sequence may be constructed using methods known in the art. For example, the nucleic acid sequence may be identified from a database, such as the GenBank or EMBL database. An expression product is produced from the nucleic acid

sequence. Changes in the growth and metabolism of an adipocyte can be measured in numerous ways, including any biological assay or visual monitoring. Nonlimiting examples of such measurements include, for example, examining a change in amount (e.g. weight) of adipose tissue, or measuring an increase in heat generation. Alternatively, the morphology of the transfected adipocytes can be compared with controls. For example, brown adipocytes have many large mitochondria and store lipid as microdroplets, whereas white adipocytes store lipid as a single large coalescent lipid droplet.

The invention may be illustrated by way of the following examples.

Example 1

In vivo gene transfer to adipocytes

Recombinant adenoviral vectors were used to transfer nucleic acid sequences in vivo to rabbit adipose tissue.

Construction of Adenoviral Vectors - An adenoviral vector containing *lacZ* cDNA coupled to the CMV promoter was prepared by the method of Spector and Samaniego, Meth. Mol. Genet., 1995, Vol. 7, p. 31-44. The *lacZ* cDNA was cloned into a shuttle plasmid containing the left end of the adenoviral genome with an E1a deletion. This plasmid also contains an origin of replication, redundant DNA encapsidation signals, and the enhancer portion of the E1a transcription control region. The shuttle plasmid and defective adenoviral DNA were co-transfected into human embryonic kidney 293 cells, and recombinant adenoviruses resolved by homologous recombination. The gene product of *lacZ*, ß-galactosidase, produces a blue color in the presence of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal).

Confirmation of gene transfer - A 3.4 kg New Zealand white rabbit was anesthetized with ketamine. A 6 cm groin incision exposed the fat pad anterior to the right hind quarter. Viral particles, 1 x 10^{11} plaque forming units (pfu), were injected in a volume of 500 μl of phosphate-buffered saline (PBS) into the fat pad. After 10 days, the animal was sacrificed and the fat pad excised and cut into approximately twelve 500 mg pieces.

ß-galactosidase activity was detected by fixing the tissue with 2% paraformaldehyde, 0.2% gluteraldehyde in PBS for 30 minutes at 4°C, then stained in the presence of 1 mg/ml X-gal for 90 minutes at 37°C. A single piece of adipose tissue, positive for ß-galactosidase activity, is shown in Figure 1. This indicates that the gene transfer was efficient and highly localized.

Example 2

Expression of MCSF in human subcutaneous adipocytes

MCSF protein was detected in subcutaneous adipocytes and adipocytes isolated from rapidly growing adipose tissue surrounding regions of inflamed bowel.

Adipocytes were isolated from a human adipose tissue biopsy. A Northern blot of 15 μg of total RNA, probed with an antisense RNA probe for base pairs 845-1460 of MCSF mRNA (GenBank, M64592), is shown in Figure 2a. No message was detected. A Western blot of protein from human adipocytes, incubated with MCSF monoclonal antibody (MAB216, R&D systems), detected MCSF protein and is shown in Figure 2b.

Reverse-transcription PCR was performed on messenger RNA extracted from human and rabbit adipocytes. After treatment of the mRNA with DNase (Gibco) for 15 minutes at 25°C, random hexamer reverse transcription with Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase generated cDNAs. Sambrook et al., (1989)

Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, NY. MCSF cDNA was amplified with 35 PCR cycles of denaturation (95°C for 30 sec), annealing (62°C for 2 minutes), and extension (72°C for 40 sec) using primers specific for human MCSF (Forward primer = GTC AAG GAC AGC ACC ATC GGT G (SEQ ID NO:1), nucleotides 805-826 of human MCSF, Reverse primer = GCT GTA CCA GTT ACA TCT GCC (SEQ ID NO:2), nucleotides 1077-1097 of human MCSF). An approximate 300 base pair product was expected from the reaction. As shown in Figure 2c, a message was detected in the human adipocytes, but not in the rabbit adipocytes. Control reactions that contained mRNA that was not reverse transcribed were negative. Amplification of cDNA from a human adipocyte library (Clontech) with the same MCSF primers also generated a product and is shown in the middle panel of Figure 2c.

Frozen human adipose tissue sections were stained for MCSF using human MCSF monoclonal antibody (MAB216, R&D Systems). Figure 2d shows the staining in the presence (i) of antibody. No staining was detected in the absence (ii) of antibody.

Mesenteric fat was obtained from two patients with Inflammatory Bowel Disease. In each case, adipose tissue was collected from a site adjacent to inflamed bowel and from a region of mesenteric fat connected to normal-appearing bowel more than 30 cm distant to the site of inflammation. Northern blot analysis (as described above) of adipocytes isolated from the samples is shown in Figure 2e. MCSF RNA was only detectable in tissue adjacent to inflamed bowel, indicating that MCSF was up-regulated in regions surrounding inflamed bowel. An immunoblot of adipocyte cellular extracts stained with Ponceau S and exposed to MCSF antibody (MAB216, R&D Systems) is shown in Figure 2f and indicates that MCSF

protein expression was up-regulated in adipose tissue surrounding regions of inflamed bowel.

Example 3

MCSF and ß-galactosidase gene transfer to subcutaneous rabbit adipocytes in vivo

Recombinant adenoviruses were used to transfer MCSF and β-galactosidase into rabbit adipose tissue in vivo.

Viral constructs - An adenoviral construct containing human MCSF cDNA (Genbank, M64592) was prepared as described in Example 1.

Experimental design - Six male New Zealand white rabbits ranging from 3.4 to 4.0 kg (mean = 3.6 kg) were used in the described gene transfer experiments. Each rabbit was anesthetized with ketamine and positioned on its back with the four limbs secured with ties. Abdominal hair was shaved, and the skin cleaned with antiseptic. Under sterile conditions, a 2 cm mid line mid-position abdominal incision was made through the skin, subcutaneous fat and fascial layers. A blunt instrument was used to dissect the left side of the abdomen to divide the fascial plane. The skin, subcutaneous fat, and fascia were then raised from abdominal musculature. A 1/3 ml insulin needle was used to determine the depth of the skin, subcutaneous fat, and fascial layer at several points in a row, 1 cm from the midline. Four blue sutures were tied in a row, 3 cm from the midline through the skin, subcutaneous fat, fascia layer, 3 cm, 5 cm, 6 cm, and 8 cm from the groin crease to create two regions of 2 cm in length boardered by sutures and divided by 1 cm.

In one region, 25 μl of 2.5×10^{10} plaque forming units (pfu) of virus particles containing the ß-galactosidase viral construct described in Example 1 were injected at a depth of 1.5 - 2 mm. The injections were

repeated four times. Four 25 μl samples of 2.5×10^{10} pfu of virus particles containing the MCSF viral construct were injected into the other region. The incision was closed and the rabbit was returned to its cage.

After five days, the procedure was repeated on the animal's right side. The relative positions of the two samples were switched.

After five more days, the animal was anesthetized and positioned on its back with its limbs tied. A pen was used to draw a 2 cm wide rectangle around the row of sutures on the left side of the animal. The rectangle extended 1 cm below and 1 cm above the lowest and uppermost sutures, respectively. The rectangle of skin, subcutaneous fat, and fascia were cut off with a scalpel and immediately placed on a plastic sheet on top of dry ice. The rectangular piece of tissue was cut across the sutures to provide two, 2 cm squares of tissue; one transfected with ß-galactosidase containing viruses and the other with MCSF containing viruses.

The two pieces of tissue were photographed and weighed. Each piece of tissue derived from each excised rectangular piece was photographed and divided into three sections for fixing in 10% formaldehyde, sectioning, and storing in liquid nitrogen. The same procedure was repeated on the right side of the animal's abdomen and the animal was sacrificed. In total, four squares of tissue were collected from each animal.

Weight of tissue squares - The weight of tissue squares (five and 10 days) from the MCSF transfected samples were compared to the ß-galactosidase controls and is shown in Table I. A 150% increase in tissue square weight was found in the five day MCSF transfected samples as compared to the ß-galactosidase controls. In the 10 day samples, the weight of the MCSF transfected samples increased 200% relative to the ß-galactosidase controls.

Extraction of lipids from the tissue samples with chloroform verified that the increased weight of the MCSF transfected samples was the result of an increased lipid content. As shown in Figure 3, a statistically significant increase in lipid content was seen in MCSF transduced adipose tissue on day 5 ($p<0.01$) and on day 10 ($p<0.0005$).

Table I

Fat Pad Weights (g) before and after induction with MCSF/virus

	DAY 5		DAY 10	
rabbit	**b-gal**	**MCSF**	**b-gal**	**MCSF**
1	2.4	4.0	2.0	6.4
2	2.5	2.9	2.4	4.7
3	1.4	2.2	1.4	3.4
4	1.6	1.5	1.2	3.8
5	1.5	2.0	1.7	3.6
6	2.7	5.4	2.9	7.9
MEAN	2.02	3.00	1.93	4.38
SD	0.58	1.46	0.64	1.23
SE	0.24	0.60	0.26	0.50

Histological evaluation of tissue squares

Hematoxylin and eosin staining was used to histologically evaluate the tissue squares. Staining was consistent with normal adipose tissue, although there was an increased number of acute and chronic inflammatory cells in some tissue sections.

Cell size of adipocytes - Cells from frozen sectioned slices of tissues were counted (n=100) to determine whether the mean cell size was different between MCSF transfected samples and the ß-galactosidase controls. No difference in mean cell size was observed.

Detection of ß-galactosidase gene expression - Sections 200 μM thick were digested with 0.5% collagenase (Sigma) for 30 minutes. Adipocytes were separated by a 5 minute, 2000g spin, fixed, then stained with X-gal as described in Example 1. The gene product of *lacZ*, ß-galactosidase, produces a blue color in the presence of X-gal. Cells were examined by light microscopy for intracellular blue staining. As shown in Figure 4, blue staining is represented by darker colored adipocytes. In ß-galactosidase transfected tissue, blue staining was present in 12.5% of the cells from day 5 and 9.3% of the cells from day 10 (Table II). No positive staining was observed in cells isolated from MCSF transfected tissue.

TABLE II

Percent blue cells after X-gal staining

	DAY 5		DAY 10	
rabbit	**b-gal**	**MCSF**	**b-gal**	**MCSF**
1	13	0	9	0
2	9	0	11	0
3	17	0	11	0
4	16	0	9	0
5	11	0	7	0
6	9	0	9	0
MEAN SE	**12.5 1.4**	**0**	**9.3 0.61**	**0**

Detection of MCSF gene expression in MCSF transduced adipose tissue - Total RNA was extracted from the tissue section stored in liquid nitrogen by guanidine thiocyanate lysis and homogenization, followed by chloroform extraction. RNA was recovered from the supernatant by applying it to an RNA Easy Column (Qiagen). After treatment with DNase (Gibco) for 15 minutes at 25°C, random hexamer reverse transcription

with MMLV reverse transcriptase was employed to generate cDNAs. Sambrook et al., (1989) Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, NY. MCSF cDNA was amplified with 35 PCR cycles of denaturation (95°C for 30 sec), annealing (62°C for 2 minutes), and extension (72°C for 40 sec) using primers specific for human MCSF (Forward primer = GTC AAG GAC AGC ACC ATC GGT G (SEQ ID NO:1), Reverse primer = GCT GTA CCA GTT ACA TCT GCC (SEQ ID NO:2)). An approximate 300 base pair product was expected from the reaction.

PCR products were separated by gel electrophoresis. Specific bands of the predicted length were excised from the gel, recovered by filtration through a spin tip, and sequenced. Comparison of the sequence obtained from the PCR reaction to the known human MCSF sequence indicated that the amplified fragment was at least 95% homologous in each of the rabbits. In the ß-galactosidase transfected samples, a band of similar size was amplified, but did not correspond to any gene in the Genbank database. It was 46.6% identical to the human MCSF in a 208 nt region. These data confirm successful transduction of rabbit adipose tissue with human MCSF.

Detection of human MCSF protein expression - Dot blots were used to detect human MCSF protein expression. Adipocyte protein was isolated from 80 mg of adipocytes (in 500 μl of water) using repeated freeze/thaw cycles and sonication. The majority of lipid was removed using floatation. The homogenate (2 μl) was applied to a nitrocellulose membrane (Protran) and blocked for 2 hours in 0.75% BSA. The blocked membrane was exposed to a monoclonal antibody specific for human MCSF (MAB216, R&D Systems) for 90 minutes then standard antimouse antibody (R&D Systems) for 45 minutes. Binding of secondary antibody was detected with ECL (Amersham) using standard

methods. The dot blot shown in Figure 5 indicates that MCSF protein was only detected in tissue inoculated with MCSF adenovirus.

Detection of adipocyte MCSF protein in MCSF transduced adipose tissue - Immunocytochemistry was used to detect intracellular MCSF expression. Slides of sectioned tissues were stained by a labeled streptavidin-biotin immunostaining procedure. Hsu, S.M. et al., 1981, J. Histochem. Cytochem., 29:577-580; Giorno, R., 1984, Diagnostic Immunol., 2:161-166. As shown in Figures 6A and 6B, MCSF protein was only detected in tissue inoculated with MCSF adenovirus.

Detection of adipocyte proliferation - Adipocyte proliferation was compared in MCSF transfected tissue with ß-galactosidase controls by immunostaining with a nuclear marker of proliferation (MIB-1) using standard methods Figure 7 shows the increase in staining in the MCSF transduced subcutaneous fat at day 10. Table III depicts the statistically significant increase in the percentage of nuclei showing positive staining in MCSF transfected tissue at both day 5 and 10.

TABLE III

Percent MIB-1 staining adipocytes

	DAY 5		DAY 10	
rabbit	b-Gal	MCSF	b-Gal	MCSF
1	2	11	2	11
2	1	6	1	16
3	3	9	0	12
4	3	18	5	15
5	2	15	3	12
6	5	12	4	10
MEAN	**2.67**	**11.83**	**2.50**	**12.67**
SE	**0.56**	**1.74**	**0.76**	**0.95**

Example 4

In vitro gene transfer to human adipocytes

Recombinant adenoviruses were shown to be capable of transferring nucleic acid sequences into human adipocytes isolated from adipose tissue biopsies.

A 5 g piece of human subcutaneous fat was cut from a surgical specimen and subjected to collagenase digestion as described in Example 3. Adipocytes were isolated by floatation as described in Example 3 and divided into two sets. In one set 35 μl of 1 x 10^{11} pfu of adenovirus containing β-galactosidase was added to 3 ml of adipocytes (1 x 10^7 cells/ml). In the control set of adipocytes, 35 μl of PBS was added. After one hour, 3 ml of DMEM (Gibco) was added to both sets of adipocytes and incubated at 37°C overnight in a slow rotating water bath. After 24 hours, the adipocytes were fixed and stained with X-gal as described in Example 1. Approximately 18% to 28% of the adenoviral treated cells were positive for β-galactosidase activity, as indicated by the dark staining in Figure 8. The control cells were negative for β-galactosidase.

Example 5

Ex vivo gene transfer to human adipocytes

Recombinant adenoviruses were shown to be capable of transferring nucleic acid sequences into human adipose tissue ex vivo.

Three 400 mg specimens of human subcutaneous fat were cut from a surgical specimen. Two of the samples were injected with 50 μl of 1 x 10^{11} pfu of β-galactosidase containing adenovirus. The third sample was injected with 50 μl of PBS. The tissue pieces were left in a 37°C incubator for 24 hours in 3 ml of DMEM (Gibco).

After 24 hours, the tissue was subjected to collagenase digestion and the adipocytes were isolated by floatation as described in Example 3. After 10 minutes of fixation in 2% paraformaldehyde, 0.2% glutaraldehyde, the cells were stained with X-gal for 90 minutes and viewed under a microscope. An example of an x-gal stained adipocyte is shown in Figure 9. Approximately 11-19% of the viral transfected cells were positive for β-galactosidase staining. The control tissue and adipocytes were negative for β-galactosidase activity.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Mayo Foundation for Medical Education and Research

(ii) TITLE OF THE INVENTION: GENE TRANSFER TO ADIPOCYTES

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C., P.A.
(B) STREET: 60 South Sixth Street, Suite 3300
(C) CITY: Minneapolis
(D) STATE: MN
(E) COUNTRY: USA
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/890,467
(B) FILING DATE: 09-JUL-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ellinger, Mark S
(B) REGISTRATION NUMBER: 34,812
(C) REFERENCE/DOCKET NUMBER: 07039/042GB1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612-335-5070
(B) TELEFAX: 612-288-9696
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCAAGGACA GCACCATCGG TG 22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGTACCAG TTACATCTGC C 21

CLAIMS

1. An in vivo method of transferring a nucleic acid sequence into an adipocyte, comprising contacting adipose tissue of a mammal with a transfer vehicle containing said nucleic acid sequence, said transfer vehicle capable of entering said adipocyte, wherein an expression product is produced from said nucleic acid sequence when transferred into said adipocyte.

2. The method of claim 1, further comprising detecting the presence of said expression product.

3. The method of claim 2, further comprising correlating the presence of said expression product with a physiologic effect in said adipocyte.

4. The method of claim 1, wherein said transfer vehicle comprises an adenovirus.

5. The method of claim 1, wherein said transfer vehicle comprises a liposome.

6. The method of claim 1, wherein said nucleic acid 6sequence encodes for macrophage colony stimulating factor or a portion thereof.

7. The method of claim 1, wherein said nucleic acid sequence is differentially regulated in adipocytes.

8. The method of claim 7, wherein said nucleic acid sequence is differentially regulated in white adipocytes versus brown adipocytes.

9. The method of claim 8, wherein said nucleic acid sequence encodes for uncoupling protein I or a portion thereof.

10. A transfected adipocyte comprising a transfer vehicle containing a nucleic acid sequence that is differentially regulated in adipocytes.

11. Adipose tissue, wherein at least one adipocyte in said adipose tissue comprises a transfer vehicle containing a nucleic acid sequence that is differentially regulated in adipocytes.

12. A method of altering adipocyte growth and metabolic regulation in a mammal comprising contacting adipose tissue of said mammal with an effective amount of a transfer vehicle containing a nucleic acid sequence, wherein said transfer vehicle is capable of entering an adipocyte of said mammal, and wherein an expression product from said nucleic acid sequence in said adipocyte alters the growth and metabolic regulation of said adipocyte.

13. The method of claim 12, wherein said nucleic acid sequence is differentially regulated in adipocytes.

14. The method of claim 12, wherein said nucleic acid sequence is differentially regulated in white adipocytes versus brown adipocytes.

15. The method of claim 14, wherein said nucleic acid sequence is uncoupling protein I.

16. A method for identifying an expression product that alters growth and metabolic regulation in an adipocyte, comprising:

a) transferring a candidate nucleic acid sequence into mammalian adipose tissue, wherein said expression product is produced from said candidate nucleic acid sequence within said adipocyte; and

b) monitoring changes in growth and metabolic regulation in said adipocyte.

17. The method of claim 16, wherein monitoring changes in adipocyte metabolism comprises examining a change in amount of adipose tissue.

The Patent Office

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Application No: GB 9814753.1
Claims searched: (1 to 17)

Examiner: Colin Sherrington
Date of search: 10 November 1998

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.P): C3H(HB7T)

Int Cl (Ed.6): A01K 67/027; C12N 5/06,5/10,15/86,15/88

Other: ONLINE: WPI,CLAIMS,DIALOG/BIOTECH

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	WO 92/06104 A1 (THE DANA-FARBER CANCER INSTITUTE & UNIVERSITY OF ILLINOIS COLLEGE OF MEDICINE) -whole document, especially page 9, line 2 to page 10, line 20; page 26, line 13 to page 31, line 19; EXAMPLES 1 to 9; claims 8 to 25	1
A	WO 95/06411 A1 (LIPOCYTE, INC.) -whole document, especially page 8, lines 19 to 23; page 9, lines 13 to 14; claims 8, 10, 12	1
A	WO 96/34100 A1 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)) -whole document	1
P, A	WO 97/49827 A2 (CORNELL RESEARCH FOUNDATION, INC.) -whole document, especially page 9, line 22 to page 10, line 7; EXAMPLE 2; claims 21 to 28	1
A	Eur.J.Biochem 1996,237,660-667 -Claire Meunier-Durmort *et al.* "Efficient transfer of regulated genes in adipocytes and heptoma cells by the combination of liposomes and replication-deficient adenovirus"	1

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

The Patent Office

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Application No: GB 9814753.1 **Examiner:** Colin Sherrington
Claims searched: (1 to 17) **Date of search:** 10 November 1998

Category	Identity of document and relevant passage	Relevant to claims
A	Proc.Natl.Acad.Sci.USA 1997,94,6386-6390 -Yan-Ting Zhou *et al.* "Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation"	1
A	Biochimica et Biophysica Acta 1997,1330,8-16 -Claire Meunier-Durmort *et al.* "Mechanism of adenovirus improvement of cationic liposome mediated gene transfer"	1

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.